Appendix A

Original Protocol
March 16, 2004

MICROBIOLOGY PROTOCOL

PROTOCOL TITLE: Fungicidal Efficacy of a Disinfectant In The Presence of Organic Soil

PURPOSE: The purpose of this study is to demonstrate the disinfectant activity of the test substance against *Trichophyton mentagrophytes*, ATCC 9533, and *Aspergillus niger*, ATCC 6275, on a representative, hard, non-porous environmental surface (i.e. sterile glass slide) at a 10-minute contact time.

SPONSOR: Reckitt Benckiser Inc., One Philips Parkway, Montvale, New Jersey 07645

TEST FACILITY: Microbiology Laboratory, Montvale Technical Center, Reckitt Benckiser Inc.
One Philips Parkway, Montvale, New Jersey 07645

PROPOSED EXPERIMENTAL START DATE: March 17, 2004

PROPOSED EXPERIMENTAL TERMINATION DATE: March 31, 2004

TEST SUBSTANCE IDENTIFICATION:

FORMULA 677-180		
Batch References	Preparation Dates (Date Pressurized)	Expiration Dates
960-028	January 28, 2004	January 2007
960-030	January 29, 2004	January 2007
960-032	January 30, 2004	January 2007

DESCRIPTION/APPLICATION OF THE TEST SUBSTANCE:
The test substance is an Aerosol Spray Disinfectant. A 2 to 3 second spray will be applied to the test surface from a distance of 6 to 8 inches for a 10-minute contact time.

DOSAGE LEVEL OF TEST SUBSTANCE:
The results of this study will be valid when the level of active ingredient(s) determined during the chemical characterization of the test substance, falls within the following specified range:

	Acceptable Levels	
Active Name	Minimum	Maximum
Ethanol	56.26	59.74%
Onyxide 3300	0.09%	0.11%

TEST SYSTEM JUSTIFICATION:

EPA Pesticide Assessment Guidelines Subdivision G: Product Performance 91-2 (e) Fungicides (pathogenic fungi) states that *Trichophyton mentagrophytes*, ATCC 9533, is the test system to be used to demonstrate the fungicidal activity of a test substance. The fungicidal activity of the test substance will also be evaluated for a second fungi, *Aspergillus niger*, ATCC 6275.

PROCEDURE FOR THE IDENTIFICATION OF THE TEST SYSTEM:

Stock cultures are logged in the Microbiology Laboratory upon receipt from the supplier (e.g. ATCC). Each individual vial is assigned a unique stock culture number, and is stored appropriately (e.g. refrigerated) until rehydrated. Upon rehydration of the stock culture, all transfers are recorded in the Culture Transfer Log, and all transfer tubes, slants or cryogenic vials are labeled with the organism name abbreviation, transfer number, and date of transfer. Examples of name abbreviations are Tm for *Trichophyton mentagrophytes* and An for *Aspergillus niger*. The detailed procedure for culture receipt, rehydration, passage and use is described in Reckitt Benckiser Microbiology/Virology SOP#4 – Stock Culture Collection and Maintenance.

ASSAY EVALUATION REQUIREMENTS:

EPA Pesticide Assessment Guidelines Subdivision G: Product Performance 91-2 (e) Fungicides (pathogenic fungi) states that the AOAC Germicidal Spray Products Test can be used to demonstrate the fungicidal activity of a test substance if the test substance is intended for use as a spray. Ten carriers on each of 2 samples representing 2 different batches must be employed in the test.

A 3rd test substance batch will be tested to satisfy the requirements of other Regulatory Agencies.

There is no statistical analysis of the test data. The EPA performance standard states that the product must kill the fungal spores on all of the carriers.

In the event that the evaluation of any of the batches demonstrates 1 true positive out of the initial 10 carriers tested, a larger number of replicates will be evaluated for that batch/organism combination. The performance standard to be applied to the follow-up evaluation of this batch will be taken from EPA Pesticide Assessment Guidelines Subdivision G: Product Performance 91-2 (d) Disinfectants (hospital or medical environment efficacy). This standard states that the product must kill the test organisms on 59 out of each set of 60 carriers per organism per batch of product.

Testing will be repeated if the growth in any of the subculture tubes is demonstrated to be a contaminant (growth that is determined not to be the test system).

ASSAY ACCEPTANCE CRITERIA:

The assay will be accepted for evaluating the test substance if the following criteria are satisfied:

1. Organisms must be recoverable from the test surface at a concentration of at least 10^4 CFU per carrier. This will be demonstrated in the Dried Recovery Control assay.
2. The neutralizer must be shown to be effective, non-toxic, and support the growth of a low number of organisms (i.e. 10-100).
3. The identity of each test system must be verified on any day it is used for testing.
4. The media, test surface and organic soil used in the study must be verified to be sterile.

TEST METHODS / REFERENCE STANDARD OPERATING PROCEDURES:

1. AOAC Germicidal Spray Products Test Method, AOAC Index, 1990, 15th edition. This method is described in detail in Reckitt Benckiser Microbiology / Virology SOP# 25.
2. Disinfectant Neutralization Confirmation. This method is described in detail in Reckitt Benckiser Microbiology / Virology SOP# 16.
3. Test Organism Survival Count. This method is described in detail in Reckitt Benckiser Microbiology / Virology SOP#17.
4. Verification of Presumptive Positive Results. This method is described in detail in Reckitt Benckiser Microbiology / Virology SOP# 53.
5. Microbiology Gram Stain Procedure. This method is described in detail in Reckitt Benckiser Microbiology / Virology SOP#58.

AOAC GERMICIDAL SPRAY TEST EXPERIMENTAL DESIGN / OPERATING TECHNIQUE

The methodology described will be performed for each of the 2 test systems.

I. TEST SYSTEM - ORGANISMS:

1. *Trichophyton mentagrophytes* (ATCC 9533)
2. *Aspergillus niger* (ATCC 4352)

II. MEDIA, REAGENTS, AND EQUIPMENT

1. Subculture / Neutralizing Broth – Letheen Broth, D/E Neutralizing Broth, or any other appropriate broth that is documented in the raw data.
2. Plating Media/Agar – Potato Dextrose Agar (PDA), or any other appropriate agar that is documented in the raw data.
3. Sterile cut glass slides, 20 x 25mm, individually contained in a glass petri dish matted with filter paper. (These slides will be used as the carriers / test surfaces).
4. Horse Serum or other appropriate blood serum.
5. 70 to 99.9% EtOH.
6. Forceps
7. Water bath(s).
8. Bunsen Burner(s).
9. Incubator(s).
10. Refrigerator(s).
11. Freezer(s).
12. -70°C Ultra Low Freezer (for stock culture storage).
13. Fluke 52 K/J Digital Thermometer
14. Rees Scientific Environmental Monitoring System.
15. Stopclock or other suitable timer that displays minutes and seconds.
16. Sterile, disposable pipettes.
17. Sterile test tubes.
18. Sterile funnels with coarse filtration medium (e.g. glass wool, cotton, gauze).
19. Sterile disposable petri dishes.

III. METHOD DESCRIPTION

A. TEST SURFACE PREPARATION (The preparation of carriers (slides) is described in detail in Reckitt Benckiser Microbiology / Virology SOP#8 – Receipt, Preparation and Handling of Chemicals, Reagents, Glassware and Carriers). Sterile glass slides will be used as the carriers / test surfaces in this assay.

The preparation of the slides will be documented in the Slide Preparation Log.

1. Clean the slides with a solution of laboratory degreasing detergent (or equivalent) and sonicated for at least 15 minutes. A ratio of 60-70 slides per 500 mL degreasing solution is recommended.
2. Rinse the slides thoroughly with Deionized water until the pH of the rinse water is measured to be between 5 – 7.
3. Using gloved hands (i.e. latex, vinyl or surgical), place the slides in a single layer onto a tray covered with paper towels and allow to dry. Once dry, visually inspect the slides for particulate debris or any residual film (e.g. excessive water stains). Any questionable slide should be discarded. In addition, the slides can wiped and/or dried with a paper towel to ensure cleanliness.
4. Using a gloved hand, place each slide into a separate petri dish matted with filter paper.
5. Sterilize the slides in an autoclave for ≥30 minutes with a drying cycle of ≥10 minutes.
6. Cool and store the slides at ambient temperature.

B. PREPARATION OF MOLD SPORE STOCK FOR TESTING

The maintenance and storage of stock cultures is described in detail in Reckitt Benckiser Microbiology/Virology SOP# 4 – Stock Culture Collection and Maintenance.

1. Subculture a cryogenic freezing bead, or an aliquot of an appropriate frozen stock, into an appropriate growth media, and incubate between 3 and 10 days. Longer incubation is acceptable.
2. After the appropriate incubation, prepare culture flasks (e.g. Sterile Cell Culture, French Square or Roux bottles or flasks) by adding 50 mL of an appropriate growth agar (e.g. Potato Dextrose, Sabouraud Dextrose) to the bottle or flask. Allow the agar to solidify. Add 1-2 mLs of the mold spore suspension from the broth tube into each bottle or flask. Spread the inoculum over the entire agar surface by using a sterile cell spreader, inoculating loop or any other appropriate sterile device. The flask or bottle can also be rocked back and forth to spread the inoculum over the agar surface.
3. Incubate the bottles or flasks at a temperature between 24 and 31°C until adequate growth is observed. This incubation time ranges from 7 to 14 days. *Aspergillus sp.* typically grows more rapidly than *Trichophyton sp.*
4. After this incubation period, add 10-15 mL of sterile buffered saline to each flask. Sterile buffered saline containing a surfactant such as 0.05% isooctylphenoxypolyethoxyethanol (Triton X-100) can also be used to resuspend the mold spores. Remove the spores (conidia) from the mycelia mats in each bottle or flask by adding 15-20 grams of 3mm sterile glass beads, and shaking the flask gently. The glass beads will assist in removing the spores from the mycelial mat. The spores can also be removed by gently rubbing the surface of the mold growth with a cell scraper or the tip of a sterile pipet.
5. Pipette the spore suspension and filter through sterile glass wool to remove any hyphal elements. If desired, the filtered suspension can be transferred to a sterile tissue grinder and ground up to break up any clumps and/or spore chains in the suspension.
6. Mold spore susspensions are kept refrigerated (2 -8°C) and are given a one month expiration date. The mold spore stock should be properly labeled (e.g Organism name, ATTC #, Culture Transfer Log Reference Number, Date Harvested, Date Expires, Storage Conditions.

C. PREPARATION OF TEST CULTURE/ADDITION OF ORGANIC LOAD

1. Determine the volume of spore suspension that will be necessary for testing purposes. Pipette an appropriate amount of the suspension into a sterile vessel and add a volume of blood serum sufficient to achieve a final serum concentration of 5% (v/v). Any combination of suspension/serum volumes which results in a final concentration of 5% serum is acceptable (e.g. 19.0 mL culture + 1.0 mL serum). This will be referred to as the TEST CULTURE.
2. Any unusual observations or problems with the test culture will be noted on the raw data sheet and reported to the Study Director immediately after it is observed or discovered. A management decision will be made as to whether to perform the study with this culture, or terminate the assay.

D. TEST SURFACE / CARRIER INOCULATION

1. Inoculate 0.01 mL of Test Culture onto each sterile slide using an appropriate micropippette.
2. Spread the Test Culture across the surface of the slide with a sterile loop or sterile wire hook.
3. It is extremely important to spread the Test Culture to the edge of the slide only, and not over onto the edge. This can result in the Test Culture drying onto an area of the slide that will not be treated with the test substance. A false positive result would then be observed.
4. Two extra slides per test system will be inoculated to serve as Dried Recovery Controls. If 2 drying times are utilized, at least one extra Dried Recovery Control carrier will be included with each drying period to demonstrate the number of organisms surviving the drying process for that drying period.

E. DRYING THE INOCULATED TEST SURFACES

1. Place the petri dishes containing the inoculated slides into a 35± 2.5°C incubator, and dry for 40 to 42 minutes. Drying temperatures outside of this range will be considered acceptable if the Dried Recovery Control values are within the acceptable range ($\geq 10^4$ organisms per carrier).
2. The slides will be visually observed at the end of the drying period to determine if the test culture is dried. If additional drying time is required, this will be documented in the raw data. Additional drying time will be deemed acceptable if the corresponding Dried Recovery Control values are within the acceptable range ($\geq 10^4$ organisms per carrier).

F. TREATMENT OF THE TEST SYSTEM (INOCULATED CARRIER) WITH THE TEST SUBSTANCE

1. The ambient temperature of the room will be recorded during the Treatment of the Test System and Subculture manipulations using an appropriate temperature monitoring device (e.g. Rees Environmental Monitoring System, Fluke Thermometer).
2. Treat an inoculated, dried test slide as per the sponsors use instructions (i.e.2-3 second spray from a distance of 6 to 8 inches).
3. At an appropriate time interval (±5 seconds), treat the next slide as described in Step 2.
4. Examples of time intervals and the maximum number of carriers that can be treated using that interval for a 10-minute contact time can be found below.
5. Continue with Steps 2 and 3 until all of the inoculated slides for that treatment set have been treated.

Examples of time intervals and maximum number of carriers that can be treated within a 10-minute contact time:

Time Interval	Maximum Number of Carriers
30 seconds	20
20 seconds	30
15 seconds	40
10 seconds	60

G. SUBCULTURE AND NEUTRALIZATION OF TREATED CARRIERS

1. Just prior to the 10-minute contact interval for the 1st slide, using an alcohol flamed and cooled forcep, remove the slide from the petri dish and drain off any excess product.
2. At the contact time (± 5 seconds), subculture the slide into a tube containing 20 mL of neutralizing media.
3. Continue to remove and subculture the remaining slides in the same order and using the same time interval as when the slides were treated.
4. After all of the treated slides have been subcultured, gently agitate or shake the entire rack of subculture tubes.
5. Subculture/transfer the slides to fresh neutralizing media if any of the following occur:
   a. The primary subculture broth becomes cloudy within 30 minutes.
   b. The neutralizing capacity of the subculture media is unknown;
   c. The neutralizing capacity of the primary subculture is known to be insufficient.

H. CONTROL – VERIFICATION OF THE IDENTITY OF THE TEST CULTURES

On the day of testing, the identity of each test system will be verified as per the instructions below. Observations from these procedures will be compared to the known characteristics of each test system. Refer to the chart provided on the next page.

1. Aseptically streak each spore suspension onto the appropriate selective media as stated in the following table:

Test System	Selective Media
Trichophyton mentagrophytes	Potato Dextrose Agar
Aspergillus niger	Potato Dextrose Agar

2. Incubate the Selective Media Agar plates between 24 and 31 °C for at least over 7 nights but no more than 14 nights. The criteria stated in the Incubation section of this protocol will apply to these test materials.
3. After incubation, examine the PDA plates for purity and type (color) of fungal growth. Record the observations.

4. Results of this verification will be reviewed by the Study Director. The identity of the test system will be Accepted or Rejected based upon the criteria provided in the table provided on the following page. This conclusion will be recorded in the raw data.

Test System	Selective Agar / Growth Characteristic
Trichophyton mentagrophytes	Potato Dextrose Agar; colonies are white in color, cottony, powdery or granular, with pale brownish pigment on the basal layer.
Aspergillus niger	Potato Dextrose Agar; growth consists of a compact white or pale yellow basal felt with a dense layer of dark brown to black conidiophores.

I. TEST SYSTEM INOCULUM / SPORE SUSPENSION COUNT

1. Dilute each test system (without organic soil) to the 10^{-7} dilution using subculture media or buffered saline.
2. Plate 1-mL aliquots of the 10^{-5}, 10^{-6} and 10^{-7} dilutions in duplicate with PDA.
3. Incubate the agar plates under the same conditions as described for the efficacy test.
4. After the appropriate incubation duration, count and record the number of colonies of each agar plate.

J. CONTROL –DRIED RECOVERY CONTROL CARRIERS

The purpose of this control is to determine the number of viable organisms that remain on the inoculated test slides after the drying period. A minimum of two slides per test organism are inoculated and dried as described for the test procedure. Recovery counts are performed on each Dried Recovery Control slide as described below. The Survivor Count procedure is described in detail in Reckitt Benckiser Microbiology / Virology SOP #17 – Test Organism Survival Count.

1. After drying, subculture each inoculated Dried Recovery Control slide into 10-mL subculture media.
2. Sonicate the tubes containing the slides for at least 5 minutes.
3. Prepare tenfold dilutions from the subculture tube containing each slide in subculture media or buffered saline. The subculture tube containing the slide is considered the 10^{0} dilution.
4. Plate 1-mL aliquots of the 10^{-2}, 10^{-3}, and 10^{-4} dilutions for each Dried Recovery Control slide with PDA.
5. Incubate the Dried Recovery Control plates under the same conditions as described in the efficacy test.
6. After the appropriate incubation duration, count the colonies on each plate. Refer to the Calculation Section of this protocol for instructions on determining the number of organisms recovered from each slide.

K. NEUTRALIZATION OF THE TEST SUBSTANCE

1. Dilute each test system (without organic soil) to the 10^{-7} dilution using subculture media or buffered saline.
2. For each slide that will be tested, a subculture tube containing 20-mL of the neutralizer / subculture media used in the efficacy assay will be inoculated with 0.1 mL of a culture dilution that will deliver 10-100 organisms into the subculture tube. (Any culture dilution that delivers 10-100 organisms can be used; 0.1 mL of either the 10^{-6} or 10^{-7} culture dilution will usually deliver the appropriate number of organisms).
3. Treat two sterile un-inoculated slides with the test substance as described in the efficacy test for each organism and culture dilution to be tested.
4. At each 10-minute contact time, subculture each un-inoculated treated slide into one of the pre-inoculated subculture tubes.
5. Confirm the number of organisms initially delivered into the subculture tubes by plating 0.1 mL of each the test culture dilution in duplicate using PDA.
6. Incubate the neutralization assay test materials under the same conditions as previously described for the efficacy test.
7. After the appropriate incubation duration, observe each subculture tube containing a slide for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media.
8. Record the result for each subculture tube as "+" for growth, or "0" for no growth.
9. Count and record the number of colonies on each agar plate.
10. Neutralization of the test substance by the neutralizer used in the efficacy study will be deemed acceptable if there is growth in both of the subculture tubes containing the treated slides, and the number of organisms introduced into each tube is determined to be between 10-100 colonies as demonstrated on the corresponding agar plates. The neutralization assay can be repeated with the same batch of subculture neutralizing media used in the efficacy test if the number of organisms is determined to be out of this acceptable range (i.e. <10 or >100).

L. CONTROL – MEDIA, TEST SURFACE AND ORGANIC SOIL STERILITY

The purpose of these controls is to verify that the media, test surface and organic soil used in the study on each assay date is sterile.

MEDIA

For liquid media (e.g. broth) at least one un-inoculated tube of each batch of liquid media used in the assay is incubated under the same conditions as stated in the efficacy assay. For agars, at least one additional plate of each batch of agar media is poured, left un-inoculated, and allowed to incubate under the same conditions as stated in the efficacy assay. Record any growth on a particular batch of media as a "NS" for non-sterile, and no growth as an "S" for sterile. If a particular batch of media demonstrates growth, and the test substance demonstrates efficacy, the identity of the test system in each of the tubes and on the plates in the Neutralizer Efficacy assay must be verified. If it is determined that the growth in the Neutralization assay is due to the test system, the assay will be accepted. A second sterility test on another tube, or agar plate can also be performed. In the event that similar growth is observed in the second sterility test,

management will be informed and a scientifically valid rationale will be developed for accepting/rejecting the results of the test using this media.

TEST SURFACE

One un-inoculated test surface (slide) will be added to a subculture tube containing the neutralizing media used in the efficacy study. A slide from each Preparation Log # used in a study will be tested for sterility. The subculture tube will be incubated under the same conditions as described previously in the efficacy assay. Record growth in the subculture tube as a "NS" for non-sterile, and no growth as an "S" for sterile. If the test surface fails (i.e. growth in subculture tube) the sterility test, and the test substance passes efficacy testing, the identity of the test system in each of the tubes and on the plates in the Neutralizer Efficacy assay must be verified. If it is determined that the growth in the Neutralization assay is due to the test system, the assay will be accepted.

ORGANIC SOIL

A 0.1-mL aliquot of the organic soil used in the study will be inoculated into a subculture tube containing the neutralizing media used in the efficacy study. The subculture tube will be incubated under the same conditions as described previously in the efficacy assay. Record growth in the subculture tube as a "NS" for non-sterile, and no growth as an "S" for sterile. If the organic soil fails (i.e. growth in subculture tube) the sterility test, and the test substance passes efficacy testing, the identity of the test system in each of the tubes and on the plates in the Neutralizer Efficacy assay must be verified. If it is determined that the growth in the Neutralization assay is due to the test system, the assay will be accepted.

M. INCUBATION

The target temperature and duration for incubation of the test materials is 24 to 31°C for 7 – 14 days.

1. Incubation at a temperature outside of this range will not be considered a protocol deviation if the Dried Recovery Control values are within the acceptable range ($\geq 10^4$ organisms per carrier). Additional instructions provided in Reckitt Benckiser Microbiology / Virology SOP 74 – Operation and Maintenance of Incubators regarding incubation temperatures and duration will be followed and adhered to if necessary.
2. Test materials removed from the incubator(s) during a weekend will be stored on the laboratory benchtop at room temperature. Results will be read on the next business day (e.g. Monday).

N. OBSERVATION OF SUBCULTURE TUBES AND RECORDING RESULTS

1. Remove the test materials from the incubator after the appropriate incubation period.
2. Observe each subculture tube containing a slide for the absence or presence of organism growth. Growth is indicated by turbidity. No growth is indicated by clear subculture media. Record each subculture tube as "+" for growth, or "0" for no growth.
3. Count the colonies on the Neutralization and Dried Recovery Control plates.

O. INVESTIGATION OF CONTAMINATION OR QUESTIONABLE GROWTH / PRESUMPTIVE POSITIVES

1. In the event the evaluation of any batch for any of the organisms tested demonstrates a presumptive positive (turbid, cloudy, tube or tube exhibiting fungal growth), an investigation of the results will be conducted as per Reckitt Benckiser Microbiology /Virology SOP #53 – Verification of Presumptive Positive Results.
2. Results of the investigation will be reviewed by the Study Director and a conclusion will be drawn as to the identity of the presumptive positive, the acceptance or rejection of the results obtained, and any necessary next steps (e.g. re-test due to the confirmed presence of a contaminant).

CALCULATIONS - INOCULUM AND DRIED RECOVERY CONTROL

1. Colony Forming Units (CFU) per ml (or organisms per ml)

   CFU/mL = A x B where

   A= Dilution Factor. The dilution factor is the inverse of the serial dilution used.
   B= Number of colonies per plate

2. CFU per Test Surface (Organisms per carrier)

CFU/Surface = A x B x C where

A= Dilution Factor. The dilution factor is the inverse of the serial dilution used.
B= Number of colonies per plate
C= Initial subculture tube volume

The initial subculture tube containing the slide is considered the 10^0 dilution.

RECORDS TO BE MAINTAINED

All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained. Raw data is entered onto the study raw data sheets, laboratory notebooks, facility log sheets and log books. Supporting records and documents that will be maintained are, but are not limited to, Product Chain of Custody, Culture Transfer, Media Production and Quality Control, and Equipment Maintenance and Calibration records.

All specimens, raw data, and the final report will be stored in the archive at the Reckitt Benckiser Inc. Technical Center, located at One Philips Parkway, Montvale, New Jersey, 07465.

PROTOCOL APPROVAL SIGNATURES:

STUDY SPONSOR: _____ DATE: 3/8/2004

STUDY DIRECTOR: Kyle L. Smith DATE: 3/16/2004

QUALITY ASSURANCE UNIT: _____ DATE: 03/18/04

QUALITY ASSURANCE UNIT MASTER SCHEDULE NUMBER: 2004 0058